SYMPOSIUM ON METABOLISM OF INORGANIC COMPOUNDS[1]

IV. Hydrogen Photosynthesis and Alternative Metabolic Pathways in Photosynthetic Bacteria[2]

JOHN G. ORMEROD[3] AND HOWARD GEST

The Henry Shaw School of Botany and the Adolphus Busch III Laboratory of Molecular Biology, Washington University, St. Louis, Missouri

I. Introduction 51
II. Photoreduction of Carbon Dioxide with Molecular Hydrogen 52
III. Photometabolism of Organic Compounds 53
A. Photoproduction of Molecular Hydrogen 54
B. The Stoichiometry of Hydrogen Production 56
C. An Anaerobic Light-dependent Citric Acid Cycle 57
D. CO_2 Fixation during Photometabolism of Citric Acid Cycle Intermediates 58
E. Alternative Pathways of Carbon Metabolism 59
IV. Functions of the Accessory Electron Donor in Bacterial Photosynthesis; Alternative Pathways of Electron Transfer 60
V. Concluding Remarks 62
VI. Addendum 63
VII. Literature Cited 63

I. Introduction

"Now although it is true that many fundamental chemical reactions are common to animals, plants and microbes, it is easy to overestimate the unity of nature when bacteria are being considered in relation to animals and plants. It is probably true to say that whilst most chemical changes occurring in the latter are either duplicated or closely paralleled in some microorganism or other, yet the converse is far from being true. The number and variety of chemical reactions already known to be catalised by bacteria far exceed those attributable to the animal and the plant together. Moreover amongst heterotrophs it is as anaerobes that bacteria specially excel; this implies that this group has elaborated special enzymes for oxidising their substrate molecules without having recourse to oxygen; in other words it is in the use of hydrogen acceptors that bacteria are specially developed as compared with animals and plants."

Marjory Stephenson, 1947 (73).

In a sense, the hydrogen-evolving enzyme present in many anaerobic microorganisms can be thought of as a special "hydrogen acceptor," with the unique capacity to combine hydrogen atoms, or electrons and hydrogen ions, to produce H_2. The ability to catalyze hydrogen formation, and to utilize H_2 as an electron donor is, in fact, surprisingly widespread among microorganisms of very different metabolic types (24), and has thus far not been observed in higher plants or animals.

There is little doubt that the electron-transfer pathways involved in production and oxidation of molecular hydrogen will prove to have many similarities with more familiar electron transport sequences in which an external acceptor is reduced or a more complex metabolite oxidized. The possession of enzymes which can evolve or oxidize H_2, however, endows many microorganisms with significantly increased potentialities for growth or for obtaining energy under unusual conditions. Thus, these variations on a basic metabolic theme have important biological implications, including a probable usefulness as "indicators" in tracing the development of biochemical evolution.

[1] This symposium was organized by Dr. Howard Gest under the auspices of the Division of General Bacteriology and presented at the Annual Meeting of the American Society for Microbiology in Chicago, Ill., on April 26, 1961. Dr. Jacques C. Senez served as honorary convener.

[2] Research of the authors supported by grants from the National Institute of Allergy and Infectious Diseases, U.S. Public Health Service (grant no. E-2640), and the National Science Foundation.

[3] Present address: Norsk Institutt for Vannforskning, Blindern, Norway.

Photosynthetic microorganisms, as a physiological group, show a remarkable degree of metabolic versatility of the kind suggested by Marjory Stephenson. In the present paper, evidence for and the significance of alternative mechanisms of light-activated electron transfer and carbon metabolism of photosynthetic bacteria are considered, in particular connection with photosyntheses in which molecular hydrogen is utilized as an electron donor or produced as a result of photometabolism.

II. Photoreduction of Carbon Dioxide with Molecular Hydrogen

For the purpose of initiating the present discussion, it is convenient to consider bacterial photosynthesis in an over-all sense as the anaerobic light-dependent conversion of carbon dioxide or organic compounds to cell materials. The utilization of CO_2 as the sole carbon source for growth by such photosyntheses depends on the presence of a suitable oxidizable compound, which we designate as the "accessory electron or hydrogen donor" (22). In 1935, Roelofsen (61) reported that molecular hydrogen could serve as the accessory donor for photoautotrophic growth of purple sulfur bacteria; other studies (10, 18, 40, 49, 50, 80) disclosed that resting suspensions of representative strains of all types of photosynthetic bacteria also can catalyze the light-dependent utilization of CO_2 in the presence of H_2.

Van Niel (76) has reported that certain nonsulfur purple bacteria can also grow photoautotrophically with H_2 as the accessory donor; a medium containing yeast extract was employed and negligible growth was observed in the absence of H_2. We considered it desirable that this observation be confirmed, particularly since recent growth experiments (47) with *Desulfovibrio desulfuricans* suggested the possibility of some uncertainty in the interpretation of Van Niel's undocumented report. In a putatively autotrophic environment containing hydrogen, bicarbonate, and limiting amounts of yeast extract, the growth of *Desulfovibrio* was observed by Mechalas and Rittenberg (47) to be dependent upon the presence of H_2, but it was also found that most of the cell carbon originated from components of the yeast extract. We have observed (Fig. 1) unambiguous growth of *Rhodospirillum rubrum* on $H_2 + CO_2$ in a completely synthetic medium, but further work is required for definition of the optimal requirements for reproducible

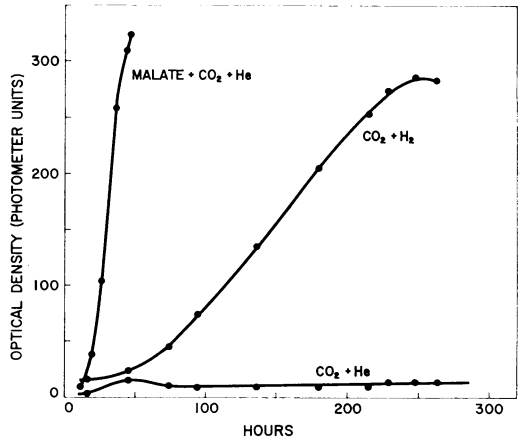


FIG. 1. *Photoautotrophic growth of Rhodospirillum rubrum (S1) on CO_2 and H_2 in a completely synthetic medium. The basal medium and conditions of incubation were similar to those described by Ormerod et al. (52) except that malate was omitted and $NaHCO_3$ added at a concentration of 0.6 mg per ml. The nitrogen source was 0.05% $(NH_4)_2SO_4$. Gas phases: 5% CO_2 + 95% H_2 or 5% CO_2 + 95% He, as indicated. The inoculum (1%) consisted of washed cells derived from a malate + $(NH_4)_2SO_4$ medium. For comparative purposes, a growth curve with 0.6% DL-malate is also shown. Optical density was measured with a Klett-Summerson photometer using filter no. 66.*

growth under these circumstances. This observation provides added evidence that separation of the photosynthetic bacteria into distinct metabolic categories is arbitrary and artificial (77).

The pathway of carbon in photoreduction of CO_2 with hydrogen has not been investigated in great detail but several studies (31, 75) suggest that the major pathways are probably very similar to those believed to be operating in other types of autotrophic CO_2 reduction. Assuming this to be the case, it might be argued that the special features of this "hydrogen photosynthesis" will be concerned with unique mechanisms by which reducing power and adenosine triphosphate (ATP) are generated.

Hydrogenase preparations from various bacteria will reduce pyridine nucleotides and flavins (36, 53, 55, 81) in the presence of H_2, and Arnon (1) has recently reported that extracts of sulfur purple bacteria, genus *Chromatium*, will similarly catalyze diphosphopyridine nucleotide (DPN) reduction in the dark, but only when supplemented with the one-electron dye benzyl

viologen. Proper evaluation of this preliminary report is difficult; data for controls in which hydrogen is replaced by an inert gas are not given and only 10% of the DPN added was reduced in an unspecified time interval. In view of these considerations, it is premature to exclude the possibility that the ultimate oxidant for H_2 in photoreduction of CO_2 is an entity generated by the photochemical apparatus.

It is known that, in certain nonphotosynthetic organisms, ATP can be generated by the oxidation of H_2 with inorganic oxidants such as oxygen (62) and sulfate (54). Thus the possibility exists that phosphorylation may also result from the anaerobic oxidation of H_2 with a photooxidant.

The properties of the hydrogenases of photosynthetic bacteria have been studied to a limited extent (23, 34, 56, 57) and are still not well understood. A start has been made on questions concerned with localization (3), and the influence of growth conditions on synthesis of the enzyme (52), but much remains to be investigated, particularly in connection with the basic problem of the fate of electrons derived from oxidation of H_2. It might be added that there is a possibility, suggested by preliminary experiments in our laboratory, that several hydrogenases with different functions may be produced by *Rhodospirillum* species.

III. Photometabolism of Organic Compounds

Purple bacteria, such as members of the genera *Rhodospirillum* and *Chromatium*, grow readily and abundantly when illuminated under anaerobic conditions in synthetic media containing malate and an ammonium salt as the sole carbon and nitrogen sources, respectively. In other words, addition of CO_2 or inorganic accessory donors is unnecessary when a suitable organic compound is provided. All of the evidence accumulated to date[4] indicates, furthermore, that purple bacteria grow much more rapidly with organic carbon sources such as C_4 dicarboxylic acids than with CO_2 (*see also* Fig. 1).

In an early investigation, Muller (48) made quantitative carbon balance studies on the utilization of a number of organic compounds by growing cultures of purple sulfur bacteria. These experiments disclosed a net production of CO_2 during growth with acetate, lactate, succinate, and malate (Table 1).

TABLE 1. *Over-all carbon dioxide metabolism in growing cultures of purple sulfur bacteria**

Substrate	Moles CO_2 produced or utilized, per mole substrate consumed†
Acetate	+0.17
Lactate	+0.29
Succinate	+0.70
Malate	+1.22
Butyrate	−0.74

* After Muller (48).

† Mean values of at least six determinations. A positive value denotes CO_2 production; negative value, CO_2 utilization.

It can be seen that the CO_2 yield varied from approximately 0.17 mole per mole of acetate used to 1.2 in the case of malate. With butyrate as the organic substrate, addition of CO_2 was required for growth and was in fact consumed to the extent of about 0.7 mole per mole of butyrate utilized. Similar results with "resting" suspensions of nonsulfur purple bacteria have been reported by other investigators (9, 17, 76). Muller interpreted his observations to indicate extensive assimilation of carbon intermediates, generated by oxidation of the organic substrate, into cell materials. Direct reductive assimilation of acetate carbon was also indicated by experiments of Gaffron (18), who demonstrated light-dependent utilization of H_2 with acetate by nonsulfur purple bacteria.

Subsequent studies, between 1935 and 1949, on the metabolism of organic compounds by purple bacteria were largely concerned with substrates (e.g., fatty acids, isopropanol) with which a net utilization of CO_2 was characteristically observed. The results of these investigations were interpreted by Van Niel (76) to support the concept that the photosynthesis of purple bacteria in the presence of organic substrates is fundamentally a photochemical CO_2 reduction with organic hydrogen donors; direct assimilation of carbon from the organic substrate was accordingly viewed as a secondary phenomenon. Comparative biochemical reasoning also led Van Niel (78) to postulate that photosynthetic CO_2 reduction could be explained on the basis of reversal of the conventional citric acid cycle.

[4] The extensive literature on metabolism of organic compounds and CO_2 by photosynthetic bacteria is discussed in a number of earlier reviews (8, 22, 29, 76); *see also* references (52) and (72).

The discovery (28) of light-dependent production of molecular hydrogen by purple bacteria as well as a number of other observations indicated the necessity for a re-evaluation of Van Niel's hypothesis. A survey of the information available in 1951 concerning the photometabolism of organic compounds led one of the present authors (22) to the following conclusions:

"The accessory (organic) 'hydrogen donor' required in bacterial photosynthesis ordinarily does not undergo a simple one-step oxidation; it generally supplies carbon intermediates, other than CO_2, which are directly used by the cell for synthetic purposes. This is apparently true for practically all classes of organic 'hydrogen donors.'"

Moreover, it was suggested (30, 33) that under conditions in which organic compounds supply intermediates for direct assimilation, photosynthetic CO_2 fixation is not obligatory and may in fact be bypassed or suppressed. Additional strong support for these views was provided by later investigations, notably by Siegel's excellent studies (65–68, 70) on the light-dependent conversion of acetone to cell materials by *Rhodopseudomonas gelatinosa*.[4]

Thus from the standpoint of carbon metabolism, the anaerobic utilization of organic compounds by purple bacteria is essentially heterotrophic in character, i.e., organic substrates are converted to intermediates which are assimilated (through syntheses requiring ATP) in the form of carbohydrate, lipid, protein, etc. In addition to this highly developed carbon heterotrophy, it is also apparent that photosynthetic bacteria possess (i) specialized systems for generation of ATP through light-activated electron transfer (11, 12, 21) and (ii) a "photosynthetic carbon cycle" presumably similar to that occurring in green plants. As has been noted previously (29), the interplay between the CO_2 reduction cycle and the heterotrophic utilization of organic compounds might be expected to result in complex patterns of electron transport as well as of carbon transformations during photosynthetic metabolism.

It can reasonably be assumed that the specific pathways used by photosynthetic bacteria for synthesis of cell materials will depend on a number of factors, which include the particular enzymatic capacities of the cell and the status of the endogenous reserves. Equally important considerations are: whether the cell is growing or resting, and the nature of the utilizable substrates provided. It seems obvious that results obtained with a given substrate and a particular organism grown under a special set of circumstances cannot be readily extrapolated to generalizations defining "the role" of organic compounds in bacterial photosynthesis. Failure, on the part of some investigators, to recognize the significance of the factors noted above has without doubt been responsible to a large degree for the tortuous history of discussions on the photometabolism of organic compounds by purple bacteria.

A. Photoproduction of Molecular Hydrogen

The occurrence of major alternative pathways in the disposition of electrons generated during photometabolism in purple bacteria is clearly indicated by the phenomenon of light-dependent production of molecular hydrogen. Purple bacteria produce large quantities of H_2 during photosynthetic growth in media containing malate as the carbon source, and molecular nitrogen or amino acids such as glutamate or aspartate serving as the nitrogen source (26–28). Resting cells derived from media of this kind rapidly produce hydrogen when illuminated in the presence of various organic compounds ((28, 30, 35); *see* Fig. 2*A*). Depending on the exact composition of the growth medium and "age" of the cells at time of harvest, photoproduction of hydrogen is sometimes observed as an endogenous process. It appears that any oxidizable substrate may evoke light-dependent H_2 production under appropriate conditions (29, 52, 69). The range of compounds effective has recently been extended by the demonstration that resting *Chromatium* cells show photoproduction of H_2, dependent upon addition of thiosulfate (45, 52). Considering the great variation in chemical nature of the substrates which have given positive results, we regard photoproduction of hydrogen as a fundamental metabolic activity intimately associated with light-activated electron transfer in all bacteria of this group.[5]

Many earlier studies, however, had indicated that molecular hydrogen was not produced dur-

[5] Green sulfur bacteria have not yet been tested, under appropriate conditions, for ability to photoproduce hydrogen. It seems very likely that such organisms will also show the phenomenon when grown under circumstances in which formation of the hydrogen-evolving system is not repressed.

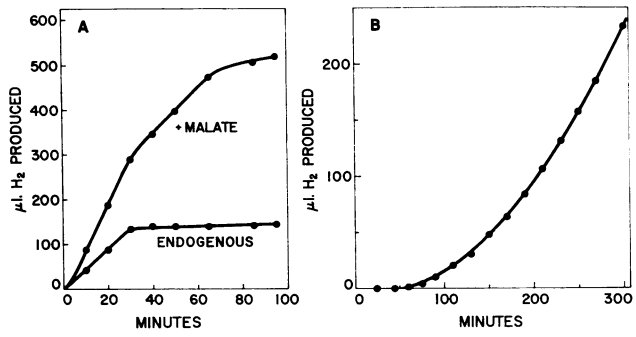


FIG. 2A (*left*). *Photoproduction of hydrogen by cells of Rhodospirillum rubrum grown on 0.6% DL-malic acid + 0.05% L-glutamic acid. Details of growth and manometric methods can be found in reference (52). The cells were harvested when the optical density of the culture was 125 Klett-Summerson units. Five micromoles of L-malate were added, where indicated, to 10 mg of cells (dry weight) at zero time. The gas phase was 1% CO_2 in He, and diethanolamine, a CO_2 buffer, was present in the center well. The Warburg vessels were illuminated at 30 C.*

FIG. 2B (*right*). *Photoproduction of hydrogen by cells of R. rubrum grown on 0.6% DL-malic acid + 0.125% $(NH_4)_2SO_4$. The cells were harvested while ammonia was still present in the culture medium (at an optical density of 240 photometer units). At zero time, 40 μmoles of L-malate were added. Other conditions as in Fig. 2A.*

ing growth of purple bacteria on malate or other organic compounds when ammonia was the nitrogen source. Resting cells from such media readily photometabolized organic substrates, but they appeared to lack the capacity to produce hydrogen. These observations led to the suggestion (26, 74) that a specific electron carrier or transport system required for hydrogen formation is not synthesized when ammonia constitutes the sole nitrogen source. Our more recent investigations (52), in which completely synthetic media were used, have disclosed that the system responsible for photoproduction of hydrogen can also develop in *Rhodospirillum* cells grown on ammonia.

Development of the hydrogen-evolving system in ammonia-grown cells requires light and a metabolizable substrate such as malate, and occurs only after ammonia has been exhausted by metabolic or other means. Cells harvested a number of hours after exhaustion of the nitrogen source from a malate medium (initially containing limiting amounts of ammonia) show immediate photoproduction of hydrogen. On the other hand, cells obtained from the medium before the ammonia has disappeared show activity only after an appreciable induction period (Fig. 2*B*). Using cells of the latter kind, experiments with inhibitors such as chloramphenicol and amino acid analogues have shown that appearance of hydrogen photoevolution activity is contingent on protein synthesis. Since addition of an external nitrogen source is unnecessary, it is likely that the amino acids required for synthesis of enzymatic components of the system can be derived from turnover of existing proteins. Addition of a small amount of glutamate, however, stimulates the development of the hydrogen-producing system, and incubation of the cells under molecular nitrogen during the induction period causes an even more pronounced acceleration (activity measured after replacement of the N_2 with helium). Our interpretation of these and related experiments (52) is that ammonia, or a metabolite derived from ammonia, represses formation of one or more specific enzymes required for hydrogen production.[6] Accordingly, this system is highly developed during growth only with nitrogen sources that do not give rise to a meas-

[6] The repression of synthesis of the hydrogen-evolving system of *R. rubrum* by ammonia is possibly another example of "feedback repression." Since ammonia can be assumed to be the end product of N_2 fixation, this interpretation would suggest that both H_2 production and N_2 activation in *R. rubrum* are catalyzed by the same enzyme or enzyme complex. Accordingly, the inhibition of photoproduction of H_2 by N_2 (28) may be due, in part, to combination of the latter gas with an enzymatic site which is also concerned with hydrogen formation.

urable steady state level of ammonia in the medium (i.e., N_2 and certain amino acids (4, 26, 27)).

From our present knowledge it seems that the operation of metabolic pathways resulting in evolution of molecular hydrogen may be the rule, rather than the exception, in the anaerobic photometabolism of *Rhodospirillum rubrum*. Considered from the standpoint of nitrogen nutrition, H_2 appears to be always produced by truly resting cells, regardless of the nitrogen growth source, and is lacking among photosynthetic growth products only when ammonia is present.

B. The Stoichiometry of Hydrogen Production

The over-all properties of the hydrogen-producing system which has developed in ammonia-grown cells appear to be generally similar to those observed with cells grown with glutamate as the nitrogen source (52). For example, in both instances the evolution of H_2 is completely light-dependent and is (reversibly) inhibited by molecular nitrogen or ammonium salts. There is, however, one significant difference between the two cell types, viz., the yields of H_2 and CO_2 observed as the result of photometabolism of a given quantity of substrate are usually much higher with glutamate-grown cells. Even with the latter nitrogen source, the particular growth conditions may have a marked influence on the quantity of hydrogen produced by the harvested organisms. This is illustrated in Fig. 3.

It is evident that as the initial concentration of glutamate in the medium is decreased, the amount of hydrogen produced by the corresponding cells increases. On the other hand, the rates of H_2 formation are relatively constant, suggesting that the hydrogen-evolving system may be developed to the same extent in each case. The data of Fig. 3 illustrate the common experience (29) that the stoichiometry of H_2 production can vary widely, depending upon the physiological state of the cells. Detailed interpretation of this particular experiment is somewhat complicated by the fact that the cell concentrations in the various cultures at the time of harvesting were significantly different; this in turn means that, due to shading, the cells were growing with somewhat different light intensities in each case and therefore could not be said to be strictly comparable. It is obvious that study of cells grown

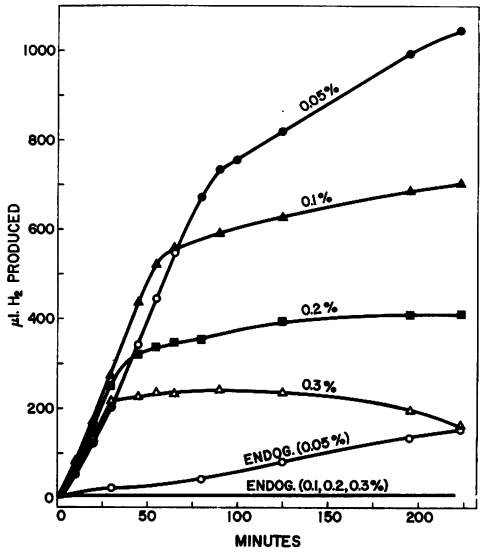


FIG. 3. *Rates and stoichiometries of photoproduction of hydrogen by cells of Rhodospirillum rubrum grown in 0.6% DL-malic acid media with different initial concentrations of L-glutamic acid. The cultures were inoculated simultaneously and harvested after 67 hr of growth in media initially containing the percentages of glutamic acid indicated. Each vessel contained 10 mg (dry weight) of cells, and 10 μ moles of L-malate were added at zero time (except for the endogenous curves). Other details as in Fig. 2A.*

in continuous culture under steady state conditions is required for a more penetrating analysis. The data at hand, however, do suggest that malate can be simultaneously utilized by at least two competitive pathways, only one of which results in hydrogen evolution.

In earlier studies on the quantitative aspects of malate photometabolism by resting cells of glutamate-grown *R. rubrum*, Bregoff and Kamen (4) found that the amount of hydrogen produced by different suspensions varied considerably. Numerous experiments, however, indicated that the stoichiometry during the early stages of metabolism could be represented by the equation:

$$C_4H_6O_5 \rightarrow \text{“}C_3H_4O_3\text{”} + CO_2 + H_2$$

where “$C_3H_4O_3$” is the assumed elementary composition of the assimilatory products. In later phases, malate was utilized at a decreased rate, whereas the rates of H_2 and CO_2 production remained constant or increased. The eventual molar ratios of H_2 and CO_2 produced, per mole

of malate consumed, were in approximate accord with the equation:

$$C_4H_6O_5 + H_2O \rightarrow \text{“}C_2H_4O_2\text{”} + 2CO_2 + 2H_2$$

Stoichiometries of this kind are suggestive of incomplete carbohydrate fermentations typical of saccharolytic *Clostridium* species, but it is also apparent that the balances observed by Bregoff and Kamen could equally well be interpreted as the resultant of two simultaneous processes: (i) conversion of part of the malate to CO_2 and a C_3 fragment, which is assimilated as carbohydrate, and (ii) extensive conversion of the remainder to CO_2 and H_2. Depending on the ratio of (i) to (ii), any one of a number of values of stoichiometry might be observed with a particular cell suspension. An increased degree of assimilation of substrate carbon into cell materials by route (i) would obviously diminish the quantity of H_2 and CO_2 produced (cf. discussion of this point by Gest, Kamen, and Bregoff, (30)).

The hydrogen yield can, in fact, exceed 2 moles per mole of malate added; yields of 3 were observed on occasion by Siegel and Kamen (69) and similar yields have been noted in the photometabolism of lactate (22). It is evidently difficult to reconcile these high yields with "normal" heterotrophic fermentation patterns.

Using the observations recorded in Fig. 3, by systematic study we have developed a procedure for obtaining cell suspensions which will rapidly photometabolize malate and succinate according to the approximate values of stoichiometry:

$$C_4H_6O_5 + 3H_2O \xrightarrow{\text{light}} 4CO_2 + 6H_2$$

$$C_4H_6O_4 + 4H_2O \xrightarrow{\text{light}} 4CO_2 + 7H_2$$

These balances indicate complete conversion to CO_2 and H_2, and, on a formal basis, suggest that at least one half of the hydrogen originates from water.

C. An Anaerobic Light-dependent Citric Acid Cycle

There is convincing evidence that the dark aerobic oxidation of organic compounds by *Rhodospirillum rubrum* occurs to a considerable extent through the conventional citric acid cycle.[4] Thus, Eisenberg (7) observed that all of the intermediates of the cycle were oxidized by cell-free extracts, and the individual steps of the cycle were demonstrated using either extracts or dried cells. Additional evidence was provided by experiments with the inhibitor fluoroacetate (9). In mammalian systems which oxidize substrates via the cycle, fluoroacetate is rapidly converted to fluorocitrate, which is a potent inhibitor of aconitase (58). Inhibition by fluoroacetate, accompanied by accumulation of citrate, is accordingly taken as strong evidence for operation of the citric acid cycle. Elsden and Ormerod (9) found that fluoroacetate caused a significant accumulation of citrate in intact cell suspensions of *R. rubrum* which were aerobically oxidizing C_4 dicarboxylic acids. Fluoroacetate greatly depressed the rate of O_2 utilization with acetate, pyruvate, propionate, and butyrate, but citrate did not accumulate in these instances, perhaps because an adequate supply of oxaloacetate was not available.

The effect of fluoroacetate on the anaerobic photometabolism of various substrates was also investigated by Elsden and Ormerod (9). In these experiments, the cells were grown with an ammonium salt as the nitrogen source, and exposed to substrates and fluoroacetate in bicarbonate buffer under an atmosphere of 5% CO_2 + 95% N_2. Since nitrogen was present in the gas phase, photoproduction of H_2 could not occur, even if the hydrogen-evolving system had been present (28, 30). Fluoroacetate caused accumulation of citrate from acetate, pyruvate, and oxaloacetate, but not from the other C_4 dicarboxylic acids. Consequently, the conclusion was drawn that the citric acid cycle was not involved in the photometabolism of succinate, fumarate, and malate. The difference in behavior of oxaloacetate and the other C_4 acids is probably related to the fact that the latter are more reduced. Thus, the reducing power resulting from oxidation of malate and its congeners to oxaloacetate, in cells which do not produce hydrogen, probably is responsible for diversion of carbon from the citric acid cycle sequence into an assimilatory pathway.

The quantitative conversion of malate to CO_2 and H_2 by glutamate-grown cells of *R. rubrum* is in net effect equivalent to the complete oxidation of malate by the conventional (aerobic) citric acid cycle. This consideration prompted experiments to test the effect of fluoroacetate on photoproduction of hydrogen. Fluoroacetate in concentrations as low as 10^{-4} M markedly inhibits evolution of H_2 in the presence of malate and it can be seen

TABLE 2. *Effect of fluoroacetate on gas production and citrate formation from L-malate by illuminated cells of Rhodospirillum rubrum*

	No fluoroacetate		$+10^{-3}$ M fluoroacetate	
	Endogenous	+5 μmoles malate	Endogenous	+5 μmoles malate
Gas (H_2 + CO_2) produced, μmoles	3.2	40.6*	0.1	8.4*
Citrate, μmoles	0.03	0.07	0.07	1.51

* These values are approximate because they do not include CO_2 bound in the liquid phase.

Experimental conditions: The cells were grown as for Fig. 2*A* and harvested when the optical density was 195 photometer units (53 hr). Each Warburg vessel contained a suspension equivalent to 1.2 mg cell nitrogen and the gas phase was 1% CO_2 in He; temperature, 30 C. Where indicated, fluoroacetate was added 15 min before addition of L-malate. Sixty-seven minutes after substrate addition, the reaction was terminated by addition of sulfuric acid. The vessel contents were then centrifuged and aliquots of the supernatant fluids were analyzed for citric acid by the pentabromoacetone method.

from Table 2 that this inhibitor also causes a massive accumulation of citrate.

These observations. together with results of experiments with other substrates to be detailed elsewhere (30a), have provided strong evidence that C_4 dicarboxylic acids and acetate can be completely converted to CO_2 and H_2 by means of an anaerobic light-dependent citric acid cycle. It is conceivable that the transformations of carbon in this process might occur through a modified cycle involving isocitratase, but this seems unlikely since the formation of this enzyme is usually under repression control and it is not present in significant quantity in cells of *R. rubrum* grown in the malate or acetate media used by Kornberg and Lascelles (38). To our knowledge, this is the first unambiguous evidence for operation of a *complete* citric acid cycle to a quantitatively significant extent under anaerobic conditions.[7]

[7] Intact cells of *Rhodospirillum rubrum* are not permeable to α-ketoglutarate and the tricarboxylic acids (7); it is therefore not possible to study the utilization of these intermediates directly. Indications suggesting operation of the anaerobic citric acid cycle during photometabolism of certain substrates can be found in data reported by Elsden and Ormerod (9), from experiments with ammonia-grown cells of *R. rubrum* incubated in the presence of N_2. It may also be remarked that an elegant, but not necessarily correct, case could be readily developed designating the anaerobic light-dependent cycle as the evolutionary predecessor of the exergonic aerobic cycle of nonphotosynthetic heterotrophs.

It is obvious that an anaerobic citric acid cycle requires a special mechanism for disposal of the electrons or hydrogen atoms released in the oxidative steps. This could be accomplished by formation of molecular hydrogen, either directly or indirectly.

D. CO_2 Fixation during Photometabolism of Citric Acid Cycle Intermediates

The anaerobic citric acid cycle might be expected to function without hydrogen production if a suitable electron acceptor is provided, or generated by the metabolism of added substrates. In this sense, CO_2 itself may act, to some extent, as an electron acceptor, after conversion to phosphoglyceric acid by the ribulose diphosphate carboxylase reaction. In Ormerod's (51) experiments with ammonia-grown cells incapable of evolving H_2 (under a N_2 atmosphere), succinate was metabolized with the net production of 0.7 mole of CO_2 and isotopic data showed that 0.5 mole of CO_2 was simultaneously fixed. This result implies (51) that the electron pair derived from oxidation of succinate to fumarate can be converted in some manner to reducing equivalents in the form of reduced pyridine nucleotide, which is then used for the reductive conversion of phosphoglyceric acid to triose phosphate.[8] However, the fact that glutamate-grown cells can convert succinate almost quantitatively to CO_2 and H_2 indicates that the succinic dehydrogenase step is not obligatorily coupled to CO_2 fixation.

It is noteworthy that Lascelles (41) has recently observed high levels of ribulose-1,5-diphosphate carboxylase in purple bacteria grown photoheterotrophically (e.g., with malate). At first glance, this finding might be considered to be somewhat unexpected, since in certain facultative autotrophs such as *Micrococcus denitrificans*

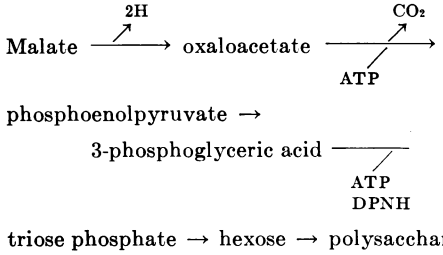
[8] It should be noted, however, that CO_2 fixation experiments of the kind under discussion are invariably complicated by the possibility of exchange reactions which would permit introduction of labeled carbon from $C^{14}O_2$ into a variety of compounds in the absence of net CO_2 fixation.

the carboxylase is present in significant quantity when the cells are grown with CO_2 as the sole carbon source, but not when assimilable organic compounds provide the growth substrates (37). From the foregoing, however, it would appear that under certain conditions purple bacteria can simultaneously fix CO_2 via the carboxylase reaction and photoassimilate fragments derived from added organic compounds. The quantitative extent of CO_2 fixation occurring through the "photosynthetic carbon cycle" during the metabolism of organic substrates is uncertain, and without doubt is strongly influenced by a variety of factors.

It is known that continuous removal of CO_2 can cause a significant decrease in the amount of H_2 evolved by purple bacteria (69), suggesting that CO_2 (or operation of the "photosynthetic carbon cycle") has the potentiality of influencing the direction of electron transfer. The possibility that this particular effect of CO_2 is catalytic cannot be excluded, especially since Fuller et al. (16) have recently described a mechanism for the conversion of malate to oxaloacetate (in *Chromatium* cells) in which CO_2 plays such a role.

E. Alternative Pathways of Carbon Metabolism

Direct analyses indicate that polysaccharide is produced as a major reserve material in *R. rubrum* during photosynthetic growth on malate, with an ammonium salt as the nitrogen source (52). Photometabolism of malate by suspensions of cells obtained from such cultures results in the formation of approximately 1 mole of CO_2 per mole of substrate added (51), when the development of the hydrogen-evolving system is prevented or its activity inhibited. These facts suggest (9) that, when H_2 is not produced, a major pathway of malate utilization is conversion to CO_2 and a C_3 fragment which is assimilated as polysaccharide, according to the following sequence:



Since the reduced pyridine nucleotide required for reduction of phosphoglyceric acid to triose phosphate could, in principle, be derived from the oxidation of malate to oxaloacetate, the only obvious net requirement is for the nucleoside triphosphate. The latter can evidently be furnished by cyclic photophosphorylation (11, 12, 21), and under these particular conditions it is possible that the necessity for light could be explained primarily on this basis.

The photoassimilatory sequence outlined above would result in the efficient conversion of approximately three of the four carbons of malate to cell materials. As in media containing ammonium salts, sizable polysaccharide reserves are also produced by *R. rubrum* during growth on malate + glutamate, presumably via the same pathway. At the same time, molecular hydrogen appears to be continuously evolved (*unpublished observations*). The control mechanisms governing the "split" of carbon flow into assimilation versus the anaerobic cycle are still not understood, but in all probability are related to the temporal energy requirements of the organism. During nonsteady-state growth, it is likely that the ATP requirement of the cell can vary significantly, depending on metabolic circumstances. For example, it can be expected that when a nitrogen source such as ammonia is exhausted, the endergonic syntheses of various macromolecular cell constituents will abruptly cease, thereby altering the demand for ATP appreciably. If it is further assumed that ATP continues to be produced under conditions of constant illumination, then it might be expected that the supply of ATP will soon exceed the diminished ATP requirement of such cells (i.e., resting cells). Thus, the partial transition from assimilation of organic intermediates to anaerobic oxidation to CO_2 and H_2 which occurs in *R. rubrum* upon exhaustion of ammonia may well be a reflection of a drastic change in "energy balance." From a physiological viewpoint, the *complete* conversion of available organic substrates to CO_2 and H_2, which can be demonstrated in resting suspensions, might be considered to be an extreme, observable only in cells of a particular physiological state. This may be so, but it is also probable that further study of such metabolically exaggerated systems may provide insights into the mechanisms of light-activated electron transfer and the nature of the metabolic control devices used by photosynthetic cells.

IV. Functions of the Accessory Electron Donor in Bacterial Photosynthesis; Alternative Pathways of Electron Transfer

Current theories concerning the action of light in photosynthesis are largely based on the premise that reducing equivalents (either electrons or hydrogen atoms) are created by the interaction of light with the photochemical apparatus, and that oxidizing equivalents (oxidized chlorophyll (Chl^+) or OH) are simultaneously produced. A description of the photochemical basis of the photoelectric theory of photosynthesis, according to which the oxidant and reductant are Chl^+ and e, respectively, can be found in papers by Levitt (42–44). The alternative postulation of H and OH as the reactive moieties generated by light is an old hypothesis (60, 63, 64, 76) and is in net effect equivalent to the photoelectric theory.[9] Light-induced electron spin resonance signals have been detected in photosynthetic systems (e.g., see reference (6)), but interpretation of these observations in terms of specific electron or hydrogen atom transfer processes is still not possible.

Detailed consideration of the available data suggests that the transport routes of photoreducing equivalents and of electrons derived from the accessory donor are not invariant, but rather that major alternative pathways are utilized, depending upon metabolic circumstances and the chemical nature of the accessory electron source. This view predicts that any attempt to explain light-activated electron transfer and associated reactions on the basis of any one specific pattern will inevitably require a number of qualifications and assumptions to cover "special cases."

The investigations of Siegel (65–68, 70) on the utilization of acetone by *Rhodopseudomonas gelatinosa* provided the first significant evidence for a partial equivalence of light and ATP in the photometabolism of organic compounds. Simultaneous-adaptation experiments with cells grown on a variety of substrates suggested the following sequence for photoassimilation of acetone:

$$\text{Acetone} \xrightarrow{+CO_2} \text{acetoacetate} \rightarrow 2\,\text{acetate} \rightarrow \text{cell materials.}$$

[9] *See* Gaffron (20) for an extensive account and critique of various alternative theories that have been proposed.

Strong supporting evidence for this pathway was obtained from experiments with C^{14}-labeled acetone and $C^{14}O_2$. Cell suspensions incubated anaerobically in the light with $C^{14}O_2$ and a mixture of unlabeled acetone and acetoacetate incorporated C^{14} into the carboxyl group of the latter compound in the predicted manner. Furthermore, at certain light intensities the accumulation of almost 2 moles of acetate, per mole of acetone used, was demonstrated. Additional experiments also indicated that the energy required ($\Delta F = +10$ kcal) for the carboxylation of acetone to acetoacetate could be derived either from light or from energy-yielding dark metabolism, viz., oxidation of acetone or fermentation of added acetoacetate. Direct experimental evidence included the demonstration that cell-free extracts of *R. gelatinosa* rapidly produce ATP from adenylic acid anaerobically in the dark, upon addition of acetoacetate and coenzyme A. Siegel interpreted his results to indicate that ATP was the common component required for the endergonic carboxylation of acetone and that this could be provided by several alternative mechanisms, viz., photophosphorylation, oxidative phosphorylation, or substrate-level phosphorylation resulting from the dark anaerobic cleavage of acetoacetate.

A more extreme version of Siegel's proposal is the recent hypothesis (46, 72) that the function of light in bacterial photosynthesis is "primarily" limited to the formation of ATP by cyclic photophosphorylation (i.e., phosphorylation resulting from the recombination of light-generated reducing and oxidizing equivalents). Reducing power (reduced pyridine nucleotide), according to this postulation, is normally produced, when required, by dark oxidation of the accessory hydrogen donor. This formulation can, *in principle*, account for several light-dependent metabolic conversions observed in photosynthetic bacteria, e.g., certain conversions which do not involve oxidation-reduction, such as the carboxylation of acetone and the synthesis of succinate from propionate + CO_2 (39), and several processes which do, viz., the formation of carbohydrate from malate and the reductive synthesis of β-hydroxybutyrate polymer from acetate (18, 72). However, its general application is seriously hampered by the lack of an unequivocal experimental basis to rationalize: (i) light-dependent reduction of DPN by chromatophores incubated in the presence of succinate or reduced flavin mononucleotide (13–15), and (ii) photoproduc-

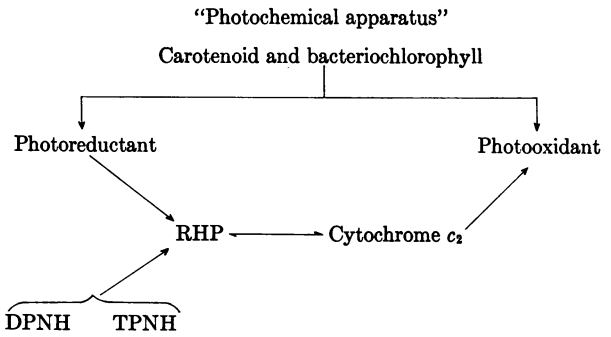


FIG. 4. *Electron transfer scheme suggested by Horio and Kamen (32). RHP is a specific heme protein present in Rhodospirillum rubrum and other photosynthetic bacteria.*

tion of H_2 from thiosulfate, C_4 dicarboxylic acids, and other organic substrates.

Clear-cut experimental evidence that electrons can be transported to an appreciable extent against the thermodynamic gradient in photosynthetic cells, through the intervention of ATP (in the dark), has not been described. According to Pratt, Frenkel, and Hickman (59), addition of ATP does not appear to promote DPN reduction by chromatophores exposed to succinate in darkness. Frenkel (14) apparently prefers to interpret the succinate-dependent photoreduction of DPN by chromatophores as the manifestation of a "noncyclic" electron transport system, in which light-generated reducing equivalents are utilized for production of reduced DPN (DPNH), balanced by transfer of electrons from succinate to the photooxidant. He has also pointed out (15) that there is as yet no definitive evidence available to permit an unambiguous choice between this interpretation and the alternative that DPNH is generated by dark oxidation of the accessory donor (facilitated by ATP), nor is there evidence to permit the assumption that only one of these mechanisms can be operative. Whether or not the photometabolism of succinate can be considered as an isolated exceptional case remains to be seen, particularly since there is so little knowledge concerning the nature of the primary acceptors involved in the oxidation of the large variety of accessory electron donors effective in bacterial photosynthesis.

The theory of complete equivalence of light and ATP (which is already undergoing revision (2) to accommodate alternative pathways) assumes, furthermore, that electrons from the accessory donor cannot enter the transport sequence normally concerned with cyclic photophosphorylation. There are, however, several indications that this may occur (14, 79). In this connection it may be noted that recent experiments by Horio and Kamen (32) were considered to be consistent with the following scheme (Fig. 4).

Photoproduction of molecular hydrogen can be considered to be analogous to the substrate-dependent photoreduction of DPN catalyzed by chromatophores. Thus, mechanisms similar to those discussed in connection with DPN photoreduction can be postulated to account for hydrogen formation. A direct "upgrading" by ATP of electrons derived from reduced pyridine nucleotide (and from oxidation of succinate to fumarate) to the level required for H_2 production is not excluded by available data,[10] but we are inclined to favor the view that photoproduction of H_2 represents a major alternative pathway of hydrogen transport, in which the photoreductant is the precursor of the electrons or hydrogen atoms of the H_2 molecule. This concept is outlined in Fig. 5; the scheme proposed is in essence the same working hypothesis which has served as a guide for experimentation for many years (*see*, e.g. (19, 25, 26)).

According to the representation in Fig. 5*B*, molecular hydrogen is produced as the result of transfer of photochemically generated electrons or hydrogen atoms to a hydrogen-evolving en-

[10] Chance (5) interprets his experiments with mammalian mitochondria to indicate that ATP-activated reversal of electron transfer may occur over a large redox potential range. It might be added that attempts to evoke H_2 production by addition of reduced pyridine nucleotides and ATP to cell-free preparations from *Rhodospirillum rubrum* have thus far given negative results (*unpublished experiments*).

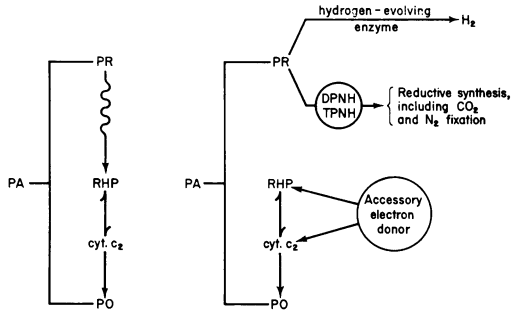


FIG. 5. *Schematic representation of (postulated) alternative light-dependent electron transfer pathways in bacterial photosynthesis. (A) represents the electron transfer chain responsible for cyclic photophosphorylation. Light-dependent phosphorylation may also occur during noncyclic electron transfer (B) when pyridine nucleotide is the acceptor. The inhibition of photoproduction of H_2 by N_2 is interpreted (26, 30) as the result of competition for photoreductant, between the nitrogen-reducing system and the hydrogen-evolving enzyme; it is assumed that the competition is greatly in favor of the former. Molecular nitrogen may also inhibit H_2 production by an additional mechanism in which N_2 acts catalytically (4). As implied by Fig. 4, electrons derived from the oxidation of accessory donors may enter the "cyclic" electron transfer chain, possibly at different points; dark oxidation of certain accessory electron donors would be expected to produce reduced pyridine nucleotides or other reduced coenzymes. PA = photochemical apparatus, PO = photooxidant (Chl^+, OH), PR = photoreductant (e, H), RHP = heme protein, cyt. c_2 = cytochrome c_2.*

zyme. An equivalent number of electrons from the oxidative steps of the citric acid cycle (or from oxidation of other accessory electron donors) are transferred through a chain of oxidation-reduction carriers to the photooxidant. This scheme indicates that a completely anaerobic citric acid cycle (without added oxidants) is made possible by two important factors. One of these is a photochemical mechanism which provides an internal oxidant for electrons released by the oxidative reactions of the cycle. This, in turn, would require continuous removal of photoreductant, which can be achieved, without the necessity for exogenous electron acceptors, by transfer to a hydrogen-evolving enzyme.

Many basic aspects of the cyclic photophosphorylation process are still unclear. Thus, the oxidation-reduction carriers involved in transport of the photoreductant to the heme protein RHP have not been identified. The yield of ATP per reducing equivalent generated by light and the actual sites of phosphorylation are also still unknown. Although it has been suggested (71) that phosphorylation may occur during electron transfer from reduced cytochrome to the photooxidant, definitive data are lacking.

Our interpretation of the mechanism and significance of photoproduction of hydrogen suggests that phosphorylation may not occur during electron transfer from RHP to the photooxidant, or as a consequence of conversion of photoreductant to H_2. This view implies that phosphorylation in cyclic electron transfer is associated with steps between the photoreductant and RHP. The possibility that phosphorylation also can occur during noncyclic electron transfer when pyridine nucleotide is the acceptor cannot be excluded (79).

V. Concluding Remarks

The calculated free energy changes associated with the conversion of citric acid cycle intermediates to CO_2 and H_2 indicate that this type of dissimilation probably cannot be a mechanism for net energy production. This suggests that photoevolution of hydrogen may represent an alternative pathway of electron transfer which serves a regulatory function in energy metabolism. Thus it could be envisioned that evolution of hydrogen is a mechanism for circumventing phosphorylation (or for utilizing excess ATP) under conditions where the cell has a diminished requirement for ATP. In analogy with current concepts of the control of electron flow and oxidative phosphorylation in aerobic systems, it might be supposed that the ratio of ATP to adenosine diphosphate in critical sites could be a key factor in determining whether the photoreductant is used as a "substrate" for cyclic photophosphorylation, for production of reduced pyridine nucleotides, or shunted to the hydrogen-evolving enzyme.

It is evident that the considerable degree of speculation inherent in current discussions of the mechanism of bacterial photosynthesis results from the fact that many basic questions remain unanswered. Nevertheless, the progress which has been made in our understanding of light-activated electron transfer and associated proc-

esses has provided the basis for a more refined definition of the basic issues and has also uncovered experimental systems of value for their study. The recognition of major alternative pathways of electron transport and carbon metabolism is clearly a prerequisite for further study of the control mechanisms governing their operation and integration, and of their true significance in the metabolic economy of the cell.

VI. Addendum

Several investigators (Frenkel (14), Vernon and Ash (79), and more recently, Nozaki, Tagawa, and Arnon (50b)) have studied "noncyclic" photo-reduction of DPN by chromatophores of *Rhodospirillum rubrum* and the relationship of this process to light-dependent phosphorylation. In the studies cited, different experimental conditions of varying complexity were used, and the data reported do not permit an unambiguous assessment of the relationship in question. It appears that clarification of this important point will require further investigation. Note should also be made of recent experiments by Nishimura (50a) which suggest that "photophosphorylation" by *R. rubrum* chromatophores can occur in darkness, i.e., during dark periods between intermittent flashes of infrared illumination.

VII. Literature Cited

1. Arnon, D. I. 1961. Cell-free photosynthesis and the energy conversion process, p. 489–565. *In* W. D. McElroy and B. Glass, [ed.], A symposium on light and life. Johns Hopkins Press, Baltimore.
2. Arnon, D. I., M. Losada, M. Nozaki, and K. Tagawa. 1961. Photoproduction of hydrogen, photofixation of nitrogen and a unified concept of photosynthesis. Nature **190**:601–606.
3. Bose, S. K., H. Gest, and J. G. Ormerod. 1961. Light-activated hydrogenase activity in a photosynthetic bacterium: a permeability phenomenon. J. Biol. Chem. **236**:PC13.
4. Bregoff, H. M., and M. D. Kamen. 1952. Studies on the metabolism of photosynthetic bacteria. XIV. Quantitative relations between malate dissimilation, photoproduction of hydrogen, and nitrogen metabolism in *Rhodospirillum rubrum*. Arch. Biochem. Biophys. **36**:202–220.
5. Chance, B. 1961. The interaction of energy and electron transfer reactions in mitochondria. II. General properties of adenosine triphosphate-linked oxidation of cytochrome and reduction of pyridine nucleotide. J. Biol. Chem. **236**:1544–1554.
6. Commoner, B., J. J. Heise, and J. Townsend. 1956. Light-induced paramagnetism in chloroplasts. Proc. Natl. Acad. Sci. U. S. **42**:710–718.
7. Eisenberg, M. A. 1953. The tricarboxylic acid cycle in *Rhodospirillum rubrum*. J. Biol. Chem. **203**:815–836.
8. Elsden, S. R. 1954. The utilization of organic compounds by photosynthetic bacteria, p. 202–223. *In* B. A. Fry and J. L. Peel, [ed.], Autotrophic micro-organisms. Cambridge University Press, Cambridge.
9. Elsden, S. R., and J. G. Ormerod. 1956. The effect of monofluoroacetate on the metabolism of *Rhodospirillum rubrum*. Biochem. J. **63**:691–701.
10. French, C. S. 1937. The quantum yield of hydrogen and carbon dioxide assimilation in purple bacteria. J. Gen. Physiol. **20**:711–735.
11. Frenkel, A. W. 1954. Light induced phosphorylation by cell-free preparations of photosynthetic bacteria. J. Am. Chem. Soc. **76**:5568–5569.
12. Frenkel, A. W. 1956. Photophosphorylation of adenine nucleotides by cell-free preparations of purple bacteria. J. Biol. Chem. **222**:823–834.
13. Frenkel, A. W. 1958. Simultaneous reduction of diphosphopyridine nucleotide and oxidation of reduced flavin mononucleotide by illuminated bacterial chromatophores. J. Am. Chem. Soc. **80**:3479–3480.
14. Frenkel, A. W. 1959. Light-induced reactions of chromatophores of *Rhodospirillum rubrum*. Brookhaven Symposia in Biol. **11**:276–287.
15. Frenkel, A. W. 1961. Reflections on recent theories concerning the mechanism of bacterial photosynthesis, p. 587–592. *In* W. D. McElroy and B. Glass, [ed.], A symposium on light and life. Johns Hopkins Press, Baltimore.
16. Fuller, R. C., R. M. Smillie, E. C. Sisler, and H. L. Kornberg. 1961. Carbon metabolism in *Chromatium*. J. Biol. Chem. **236**: 2140–2149.
17. Gaffron, H. 1933. Über den Stoffwechsel der schwefelfreien Purpurbakterien. Biochem. Z. **260**:1–17.
18. Gaffron, H. 1935. Über den Stoffwechsel der Purpurbakterien. II. Biochem. Z. **275**:301–319.
19. Gaffron, H. 1944. Photosynthesis, photoreduction and dark reduction of carbon dioxide in certain algae. Biol. Rev. Cambridge Phil. Soc. **19**:1–20.

20. GAFFRON, H. 1960. Energy storage: photosynthesis, p. 3–277. *In* F. C. Steward, [ed.], Plant physiology, v. IB. Academic Press, Inc., New York.
21. GELLER, D. M., AND F. LIPMANN. 1960. Photophosphorylation in extracts of *Rhodospirillum rubrum*. J. Biol. Chem. **235**:2478–2484.
22. GEST, H. 1951. Metabolic patterns in photosynthetic bacteria. Bacteriol. Rev. **15**:183–210.
23. GEST, H. 1952. Properties of cell-free hydrogenases of *Escherichia coli* and *Rhodospirillum rubrum*. J. Bacteriol. **63**:111-121.
24. GEST, H. 1954. Oxidation and evolution of molecular hydrogen by microorganisms. Bacteriol. Rev. **18**:43–73.
25. GEST, H. 1958. Some aspects of electron transport in microorganisms with special reference to oxidation and formation of molecular hydrogen, p. 250–255. *In* K. Ichihara, [ed.], Proceedings of the international symposium on enzyme chemistry (Tokyo and Kyoto, 1957). Academic Press, Inc., New York.
26. GEST, H., J. JUDIS, AND H. D. PECK, JR. 1956. Reduction of molecular nitrogen and relationships with photosynthesis and hydrogen metabolism, p. 298–315. *In* W. D. McElroy and B. Glass, [ed.], Inorganic nitrogen metabolism. Johns Hopkins Press, Baltimore.
27. GEST, H., AND M. D. KAMEN. 1949. Studies on the metabolism of photosynthetic bacteria. IV. Photochemical production of molecular hydrogen by growing cultures of photosynthetic bacteria. J. Bacteriol. **58**:239–245.
28. GEST, H., AND M. D. KAMEN. 1949. Photoproduction of molecular hydrogen by *Rhodospirillum rubrum*. Science **109**:558–559.
29. GEST, H., AND M. D. KAMEN. 1960. The photosynthetic bacteria, p. 568–612. *In* W. Ruhland, [ed.], Encyclopedia of plant physiology, v. V/2. Springer-Verlag, Berlin-Göttingen-Heidelberg.
30. GEST, H., M. D. KAMEN, AND H. M. BREGOFF. 1950. Studies on the metabolism of photosynthetic bacteria. V. Photoproduction of hydrogen and nitrogen fixation by *Rhodospirillum rubrum*. J. Biol. Chem. **182**:153–170.
30a. GEST, H., J. G. ORMEROD, AND K. S. ORMEROD. 1962. Photometabolism of *Rhodospirillum rubrum:* light-dependent dissimilation of organic compounds to carbon dioxide and molecular hydrogen by an aerobic citric acid cycle. Arch. Biochem. Biophys. (*in press*).
31. GLOVER, J., M. D. KAMEN, AND H. VAN GENDEREN. 1952. Studies on the metabolism of photosynthetic bacteria. XII. Comparative light and dark metabolism of acetate and carbonate by *Rhodospirillum rubrum*. Arch. Biochem. Biophys. **35**:384–408.
32. HORIO, T., AND M. D. KAMEN. 1960. Some haem protein-linked pyridine nucleotide oxidation systems in *Rhodospirillum rubrum*. Biochim. et. Biophys. Acta **43**:382–392.
33. KAMEN, M. D. 1950. Hydrogenase activity and photoassimilation. Federation Proc. **9**: 543–549.
34. KARUNAIRATNAM, M. C., AND H. GEST. 1958. Hydrogenase and photometabolism in *Rhodospirillum rubrum*. Abstr., Intern. Congr. Microbiol., 7th Congr. (Stockholm), p. 74.
35. KOHLMILLER, E. F., JR., AND H. GEST. 1951. A comparative study of the light and dark fermentations of organic acids by *Rhodospirillum rubrum*. J. Bacteriol. **61**:269–282.
36. KORKES, S. 1955. Enzymatic reduction of pyridine nucleotides by molecular hydrogen. J. Biol. Chem. **216**:737–748.
37. KORNBERG, H. L., J. F. COLLINS, AND D. BIGLEY. 1960. The influence of growth substrates on metabolic pathways in *Micrococcus denitrificans*. Biochim. et Biophys. Acta **39**:9–24.
38. KORNBERG, H. L., AND J. LASCELLES. 1960. The formation of isocitratase by the *Athiorhodaceae*. J. Gen. Microbiol. **23**:511–517.
39. LARSEN, H. 1951. Photosynthesis of succinic acid by *Chlorobium thiosulphatophilum*. J. Biol. Chem. **193**:167–173.
40. LARSEN, H. 1953. On the microbiology and biochemistry of the photosynthetic green sulfur bacteria. F. Bruns Bokhandel, Trondheim. 199 pp.
41. LASCELLES, J. 1960. The formation of ribulose 1:5-diphosphate carboxylase by growing cultures of *Athiorhodaceae*. J. Gen. Microbiol. **23**:499–510.
42. LEVITT, L. S. 1953. Photosynthesis as a photoelectric phenomenon. Science **118**:696–697.
43. LEVITT, L. S. 1954. The role of magnesium in photosynthesis. Science **120**:33–35.
44. LEVITT, L. S. 1960. The photoelectric theory of photosynthesis. V. Further correlation of the absorption spectrum of chlorophyll with the emission spectrum of magnesium. Appl. Spectroscopy **14**: 161–164.
45. LOSADA, M., M. NOZAKI, AND D. I. ARNON. 1961. Photoproduction of molecular hydrogen from thiosulfate by *Chromatium* cells, p. 570–575. *In* W. D. McElroy and B. Glass, [ed.], A symposium on light and life. Johns Hopkins Press, Baltimore.
46. LOSADA, M., A. V. TREBST, S. OGATA, AND D.

I. ARNON. 1960. Equivalence of light and adenosine triphosphate in bacterial photosynthesis. Nature **186**:753–760.

47. MECHALAS, B. J., AND S. C. RITTENBERG. 1960. Energy coupling in *Desulfovibrio desulfuricans*. J. Bacteriol. **80**:501–507.
48. MULLER, F. M. 1933. On the metabolism of the purple sulphur bacteria in organic media. Arch. Mikrobiol. **4**:131–166.
49. NAKAMURA, H. 1937. Über die Photosynthese bei der schwefelfreien Purpurbakterie, *Rhodobacillus palustris*. Beiträge zur Stoffwechselphysiologie der Purpurbakterien, I. Acta Phytochim (Japan) **9**:189–229.
50. NAKAMURA, H. 1938. Über die Rolle der Hydrogenase im Stoffwechsel von *Rhodobacillus palustris*. Beiträge zur Stoffwechselphysiologie der Purpurbakterien, IV. Acta Phytochim. (Japan) **10**:259–270.

50a. NISHIMURA, M. 1961. Photophosphorylation by intermittent light. Federation Proc. **20**: 374.

50b. NOZAKI, M., K. TAGAWA, AND D. I. ARNON. 1961. Noncyclic photophosphorylation in photosynthetic bacteria. Proc. Natl. Acad. Sci. U. S. **47**: 1334–1340.

51. ORMEROD, J. G. 1956. The use of radioactive carbon dioxide in the measurement of carbon dioxide fixation in *Rhodospirillum rubrum*. Biochem. J. **64**:373–380.
52. ORMEROD, J. G., K. S. ORMEROD, AND H. GEST. 1961. Light-dependent utilization of organic compounds and photoproduction of molecular hydrogen by photosynthetic bacteria; relationships with nitrogen metabolism. Arch. Biochem. Biophys. **94**: 449–463.
53. PACKER, L., AND W. VISHNIAC. 1955. The specificity of a diphosphopyridine nucleotide-linked hydrogenase. Biochim. et Biophys. Acta **17**:153–154.
54. PECK, H. D., JR. 1960. Evidence for oxidative phosphorylation during the reduction of sulfate with hydrogen by *Desulfovibrio desulfuricans*. J. Biol. Chem. **235**:2734–2738.
55. PECK, H. D., JR., AND H. GEST. 1954. Enzymic reduction of pyridine nucleotides by molecular hydrogen. Biochim. et Biophys. Acta **15**:587–588.
56. PECK, H. D., JR., AND H. GEST. 1956. A new procedure for assay of bacterial hydrogenases. J. Bacteriol. **71**:70–80.
57. PECK, H. D., JR., A. SAN PIETRO, AND H. GEST. 1956. On the mechanism of hydrogenase action. Proc. Natl. Acad. Sci. U. S. **42**:13–19.
58. PETERS, R., R. W. WAKELIN, P. BUFFA, AND L. C. THOMAS. 1953. Biochemistry of fluoroacetate poisoning. The isolation and some properties of the fluorotricarboxylic acid inhibitor of citrate metabolism. Proc. Roy. Soc. (London), B, **140**:497–507.
59. PRATT, D. C., A. W. FRENKEL, AND D. D. HICKMAN. 1961. Observations on the formation of the photosynthetic apparatus in *Rhodospirillum rubrum* and some comments on light-induced chromatophore reactions. *In* Biological structure and function. First IUB/IUBS Joint Symposium (Stockholm, 1960), (*in press*).
60. RABINOWITCH, E. I. 1945. Photosynthesis and related processes, p. 155. Interscience Publishers, Inc., New York.
61. ROELOFSEN, P. A. 1935. On photosynthesis of the *Thiorhodaceae*. Doctoral thesis, Univ. of Utrecht, Utrecht, Netherlands.
62. ROSE, I. A., AND S. OCHOA. 1956. Phosphorylation by particulate preparations of *Azotobacter vinelandii*. J Biol. Chem. **220**:307–314.
63. SHIBATA, K. 1931. Carbon and nitrogen assimilation (in Japanese). Iwanami Publishing Co., Hitotsubashi-Kanda-Tokyo. 65 pp.
64. SHIBATA, K., AND E. YAKUSHIJI. 1933. Der Reaktionsmechanismus der Photosynthese. Naturwissenschaften **21**:267–268.
65. SIEGEL, J. M. 1950. The metabolism of acetone by the photosynthetic bacterium *Rhodopseudomonas gelatinosa*. J. Bacteriol. **60**: 595–606.
66. SIEGEL, J. M. 1954. The photosynthetic metabolism of acetone by *Rhodopseudomonas gelatinosa*. J. Biol. Chem. **208**:205–216.
67. SIEGEL, J. M. 1956. The application of carbon-14 to studies on bacterial photosynthesis. Proc. Intern. Conf. Peaceful Uses Atomic Energy **12**:334–339.
68. SIEGEL, J. M. 1957. The dark anaerobic metabolism of acetone and acetate by the photosynthetic bacterium *Rhodopseudomonas gelatinosa*. J. Biol. Chem. **228**:41–47.
69. SIEGEL, J. M., AND M. D. KAMEN. 1951. Studies on the metabolism of photosynthetic bacteria. VII. Comparative studies on the photoproduction of H_2 by *Rhodopseudomonas gelatinosa* and *Rhodospirillum rubrum*. J. Bacteriol. **61**:215–228.
70. SIEGEL, J. M., AND A. A. SMITH. 1955. The dark aerobic metabolism of acetone by the photosynthetic bacterium *Rhodopseudomonas gelatinosa*. J. Biol. Chem. **214**:475–482.
71. SMITH, L., AND M. BALTSCHEFFSKY. 1959. Respiration and light-induced phosphorylation in extracts of *Rhodospirillum rubrum*. J. Biol. Chem. **234**:1575–1579.
72. STANIER, R. Y., M. DOUDOROFF, R. KUNISAWA, AND R. CONTOPOULOU. 1959. The role of organic substrates in bacterial photosyn-

thesis. Proc. Natl. Acad. Sci. U. S. **45**:1246–1260.

73. STEPHENSON, M. 1947. Some aspects of hydrogen transfer. Antonie v. Leeuwenhoek. J. Microbiol. Serol. **12**:33–48.
74. STIFFLER, H. J., AND H. GEST. 1954. Effects of light intensity and nitrogen growth source on hydrogen metabolism in *Rhodospirillum rubrum*. Science **120**:1024–1026.
75. STOPPANI, A. O. M., R. C. FULLER, AND M. CALVIN. 1955. Carbon dioxide fixation by *Rhodopseudomonas capsulatus*. J. Bacteriol. **69**:491–501.
76. VAN NIEL, C. B. 1941. The bacterial photosyntheses and their importance for the general problem of photosynthesis. Advances in Enzymol. **1**:263–328.
77. VAN NIEL, C. B. 1944. The culture, general physiology, morphology, and classification of the non-sulfur purple and brown bacteria. Bacteriol. Rev. **8**:1–118.
78. VAN NIEL, C. B. 1949. The comparative biochemistry of photosynthesis, p. 437–495. *In* J. Franck and W. E. Loomis, [ed.], Photosynthesis in plants. Iowa State College Press, Ames.
79. VERNON, L. P., AND O. K. ASH. 1960. Coupled photooxidation and photoreduction reactions and associated phosphorylation by chromatophores of *Rhodospirillum rubrum*. J. Biol. Chem. **235**:2721–2727.
80. WASSINK, E. C. 1942. On the ratio between the uptake of carbon dioxide and of the hydrogen donor in purple sulphur bacteria. Enzymologia **10**:257–268.
81. WITTENBERGER, C. L., AND R. REPASKE. 1961. Studies on hydrogen oxidation in cell-free extracts of *Hydrogenomonas eutropha*. Biochim. et Biophys. Acta **47**:542–552.